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STUDIES IN EXPERIMENTAL LATHYRISM—IV ON RECONSTITUTION OF NEUTRAL SALT-SOLUBLE COLLAGEN FROM LATHYRITIC RATS

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Abstract—The 'water gel' formation from the neutral salt-soluble collagen of lathyritic rats was retarded in comparison to a respective normal sample. The 'reconstitution' of denatured, lathyritic collagen was also disturbed, depending on the duration of the denaturation and on the temperature at the 'reconstitution'.

IN LATHYRISM there is a defect in the maturation of the soluble forms of collagen into insoluble fibres.[1, 2] The purpose of these experiments was to explore, whether any irregularity could be demonstrated in the aggregation of the tropocollagen molecules[3] 'end-to-end' at dialysis against water, in their aggregation 'side-to-side' by precipitation with ATP and, finally, whether the 'reconstitution' of denatured collagens[4, 5] was normal in lathyrism.

EXPERIMENTAL

Preparation of samples

Neutral salt-soluble collagen was extracted at +5° from minced, fresh skins of normal and lathyritic rats with 0·45 M sodium chloride in 1/15 M phosphate buffer, pH 7·4. The solutions were cleared by centrifugation with MSE centrifuge at 35,000*g* for 1 hr at +5°. The samples were purified by precipitation with sodium chloride (final concentration 16% w/v). The dissolution, precipitation and centrifugation was repeated three times. The samples were stored in lyophilized form. The experimental solutions were made in 0·5% acetic acid and dialyzed against sodium citrate buffer, pH 3·7, containing 0·075 M of sodium ion. Following samples were used:

KB, normal, contained 0·094 per cent collagen;
KC, normal, contained 0·189 per cent collagen (according to an ultracentrifuge diagram 0·222 per cent);
LB, lathyritic, contained 0·100 per cent collagen;
LC, lathyritic, contained 0·230 per cent collagen (according to an ultracentrifuge diagram 0·252 per cent).

The collagen concentrations were calculated from the hydroxyproline contents[6] (multiplication factor 7·15). The concentrations were adjusted so that the relative viscosities of the respective normal and lathyritic samples were identical. The specific viscosities of the lathyritic collagen samples were at these concentrations slightly lower than those of the normal samples.

Dialysis against water

The samples KB, KC, LB and LC were first dialyzed overnight against 0·5% acetic acid solution and thereafte rat +5·0° against distilled water, which was changed daily as shown in Fig. 1. The viscosity of the solutions was estimated from the flow time from a pipet (the flow time of water at +5.0° 1·75 sec). The flow time was at every measurement initially somewhat higher but decreased then to constant values, shown in Fig. 1.

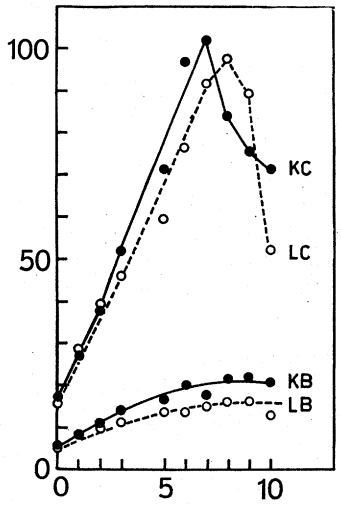

FIG. 1. The formation of 'water gel' from salt-soluble collagens.
The specific viscosity $(\eta_{rel}-1)/c$ of the inside fluid is plotted against the duration of the experiment (abscissa, in days). The outside fluid was changed daily, except on the fourth day. KB, KC are normal and LB, LC lathyritic samples, explained in the text.

Precipitation with ATP

Free ATP was prepared from its sodium salt using CG-50 Amberlite column, which was eluted with 0·05% acetic acid. Thus a 0·2% solution of ATP in 0·05% acetic acid was obtained. The collagen solutions (KB and LB, dialyzed at +5° against 0·05% acetic acid solution for 4 days) were denatured at +37·0° for 0, 5, 10, 20 and 30 min and allowed to reaggregate at +5° for 13 hr. The ATP solution was

TABLE 1. FRACTION OF TOTAL SOLUBLE COLLAGEN, NOT PRECIPITABLE WITH ATP

Duration of denaturation, min	Not precipitable, per cent of total			
	Control samples		Lathyritic samples	
	I	II	I	II
0	0·5	0·5	0	0
5	2·1	1·3	0·4	0·2
10	8·3	7·5	3·9	3·1
20	11·7	10·0	5·8	6·0
30	11·4	10·8	8·6	8·8

I and II indicate two independent series of experiments.

added, half the volume of the collagen solution. After standing for 30 hr at +5°, the precipitate was removed by centrifugation for 5 min at 18,000 rev/min and hydroxyproline was determined[7] from the supernatants. The results are shown in Table 1. The total hydroxyproline concentration in the samples varied 103–135 μg/ml.

'Reconstitution' of denatured collagen

The changes were followed with an Ostwald type viscosimeter. The flow time with the mentioned 0·075 M citrate buffer, pH 3·7, was at + 5·0° 61·1 sec, at + −20·0° 39·7 sec and at +37·0° 27·9 sec.

The samples were first denatured at +37° 15–60 min and then transferred to +5° or +20° and the increase of the viscosity was recorded. Also the course of denaturation was followed with viscosimetry in some cases but the control and lathyritic samples did not differ in this respect.

The data are collected to Fig. 2 and Table 2.

RESULTS

Obviously a stronger 'water gel' was formed from the control than from the lathyritic sample (Fig. 1). At the peak of the curves a turbidity appeared because of the precipitation of collagen, which explains the abrupt decrease in the viscosity. The precipitation with ATP showed that, even after denaturation, the precipitation is quite complete, and surprisingly, more complete in lathyritic samples (Table 1). This could be interpreted as an evidence for a different 'charge profile' in partially denatured lathyritic collagen.

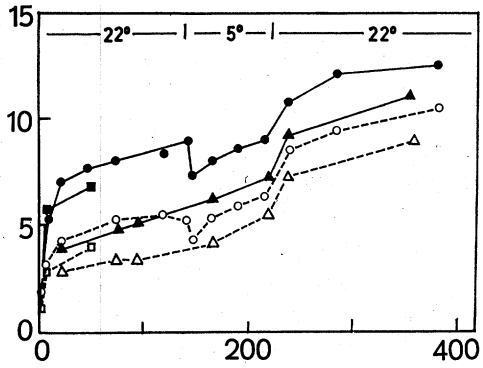

FIG. 2. The increase of the viscosity of denatured collagen solutions during the storage. The specific viscosities $(\eta_{rel}-1)/c$ are plotted against the duration of the experiment (abscissa, hr). Closed symbols indicate samples from normal and open symbols the respective samples from lathyritic rats. The samples shown with triangles were denatured for 60 min, those indicated with circles or squares for 30 min.

The reconstitution experiments (Fig. 2, Table 2) showed convincingly that, at least in the terms of viscosity, the normal collagen reconstituted at a higher rate than lathyritic. The difference between normal and lathyritic collagen was larger, if the denaturation was of shorter duration and presumably less complete, and if the reconstitution was allowed to take place at +20°.

TABLE 2. 'RECONSTITUTION' OF DENATURED SOLUBLE COLLAGENS.

KB, KC normal and LB, LC lathyritic samples, explained in the text. The viscosities are expressed as 'reduced viscosity' $\eta_{rel}-1$. The concentrations remained constant during the denaturation and 'reconstitution'. The 'final' viscosity at 'reconstitution' means the value after 400 hr.

Sample	Initial		Denaturation		Reconstitution		
	Temperature, °C	Viscosity	Duration, min	Final viscosity	Temperature, °C	Final viscosity	Effect of lathyrism, per cent
KB	5	2·78	60	0·046	5	0·170	
LB	5	2·76	60	0·046	5	0·154	−9
KB	5	2·96	30	0·095	5	0·258	
LB	5	3·02	30	0·086	5	0·220	−15
KB	5	2·88	15	—	5	0·383	
LB	5	2·97	15	—	5	0·310	−19
KC	5	11·6	60	0·142	5	1·00	
LC	5	11·1	60	0·140	5	1·05	+5
KC	5	11·4	30	0·196	5	1·17	
LC	5	11·5	30	0·186	5	1·16	−1
KC	5	11·0	15	0·402	5	1·53	
LC	5	11·1	15	0·402	5	1·42	−7
KB	20	2·83	30	—	20	0·638	
LB	20	2·81	30	—	20	0·412	−35
KC	20	11·0	30	—	20	2·55	
LC	20	10·8	30	—	20	1·97	−22
KE†	20	7·3	30	0·113	20	1·27	
LE†	20	7·0	30	0·113	20	1·00	−21

* $P < 0·02$, if the data are treated as non-independent pairs.
† Same materials as KB, KC and LB, LC, but concentrations unknown.

DISCUSSION

We think that these experiments contribute to the evidence for an organizational defect in lathyrism on the macromolecular level. Because the difference in 'reconstitution' depends on the duration of denaturation, which is known to proceed in two phases[8], it is suspected that in lathyrism there is also a disturbance in the rate of denaturation. This difference would influence the 'reconstitution', but it was not reflected in the viscosity during the denaturation.

Further comments are not warranted because (1) the 'reconstitution' in terms of viscosity can depend on many kinds of macromolecular changes and also (2) because the normal and lathyritic salt-soluble collagen samples are not identical by subunit composition.[1, 2] It is known that the denaturation depends definitely on the presence of γ-components.[4] Only α_1- and α_2-components could be demonstrated in the heat-denatured lathyritic sample by starch gel electrophoresis (performed by Drs. Näntö and Maatela[9]), but the denatured normal sample contained also the β-components. Therefore the question remains open, whether the observed differences depend on the subunit composition or on some change in the α-subunits themselves.

Work is in progress to settle this question with isolated subunits. If the mixtures of α_1- and α_2-units from normal and lathyritic collagen 'reconstitute' similarly, the defect should be sought in the rates of formation of β_1- and β_2-units *in vitro* and *in vivo*. If

the formation of the β-units is defective in lathyrism, it could be concluded that this step is a necessary, and sometimes limiting, premise for the normal organization of soluble collagen to final insoluble fibres. Gross[10] studied the reversibility of heat gelation of collagen on cooling to $+3°$ after various periods of incubation at $+37°$ and found that the lathyritic sample was much more soluble at cooling. He suggests that 'the mechanism might involve a subtle alteration in collagen structure or interference by another firmly bound substance'. Similar observations on gelling and time-dependent solubility changes of lathyritic collagen have been reported by Hausmann.[11]

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REFERENCES

1. G. R. Martin, J. Gross, K. A. Piez and M. S. Lewis, *Biochim. Biophys. Acta* **53**, 599 (1961).
2. T. Nikkari and E. Kulonen, *Biochem. Pharmacol.* **11**, 931 (1962).
3. F. O. Schmitt, in *Connective Tissue, Thrombosis and Atherosclerosis*, ed. by I. H. Page, p. 43, Acad. Press 1959.
4. K. Altgelt, A. J. Hodge and F. O. Schmitt, *Proc. nat. Acad. Sci. U.S.A.* **47**, 1914 (1961).
5. A. Veis, J. Anesey and J. Cohen, *Arch. Biochem. Biophysics* **94**, 20 (1961).
6. R. E. Neuman and M. A. Logan, *J. Biol. Chem.* **184**, 299 (1958).
7. J. F. Woessner Jr., *Arch. Biochem. Biophys.* **93**, 440 (1961).
8. J. Engel, *Arch. Biochem. Biophys.* **97**, 150 (1962).
9. V. Näntö, J. Maatela and E. Kulonen, *Acta Chem. Scand.*, in Press.
10. J. Gross, *Biochim. Biophys. Acta* **71**, 250 (1963).
11. E. Hausmann, *Fed. Proc.* **22**, 191 (1963).